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Characterization of an outward rectifying chloride current of *Xenopus tropicalis* oocytes

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ABSTRACT

Here, we describe an outward rectifying current in *Xenopus tropicalis* oocytes that we have called xtClC-or. The current has two components; the major component is voltage activated and independent of intracellular or extracellular Ca^{2+}, whereas the second is a smaller component that is Ca^{2+} dependent. The properties of the Ca^{2+}-independent current, such as voltage dependence and outward rectification, resemble those of ClC anion channels/transporters. This current is sensitive to NPPB and NFA, insensitive to 9 AC and DIDS, and showed a whole-cell conductance sequence of $SCN^- > I^- > Br^- > Cl^-$. RT-PCR revealed the expression in oocytes of ClC-2 to ClC-7, and major reductions of current amplitudes were observed when a ClC-5 antisense oligonucleotide was injected into oocytes. The Ca^{2+}-dependent component was abated after injection of 10 mM BAPTA or EGTA, whereas 10 mM Mg^{2+} inhibited the current to 26 ± 3.1%. This component was blocked by 9-AC, NFA, and NPPB, whereas DIDS did not elicit any evident effect. The ion sequence selectivity was $SCN = I^- > Br^- > Cl^-$. To try to determine the molecular identity that gives rise to this component we assessed by RT-PCR the expression of the Ca^{2+}-dependent Cl^- channel TMEM16A, which was found to be present in the oocytes. However, injection of antisense TMEM16A oligonucleotides did not inhibit the transient outward current. This result fits well with the electrophysiological data. Together, these results suggest that ClC-5 is a major, but not the sole channel responsible for this outwardly rectifying Cl^- current.

1. Introduction

Amphibian oocytes exhibit a variety of ion conductances, including several selective for Na^+, K^+, Ca^{2+}, and Cl^- that are activated by different mechanisms such as voltage changes, mechanical stimuli, or variations in the levels of intracellular Ca^{2+}. Anion channels of oocytes include bestrophins and TMEM16A, which are Ca^{2+}-dependent Cl^- channels (CaCC) [1,2], and voltage-dependent Cl^- channels/transporters such as ClCs [3]. However, the contribution of these channels to the control of the plasma membrane resting potential or even their relation to previously characterized endogenous currents is not yet well known.

Xenopus laevis oocytes used as models for studying ion-channel function have provided knowledge fundamental to understanding general mechanisms of cell physiology. On the other hand, recent completion of the genome sequence of *Xenopus tropicalis* [4] makes this species an alternative model for studying fundamental questions about the role of ion channels and receptors during oogenesis and early development. In assessing the utility of *X. tropicalis* oocytes for experimental studies in this area, it is important to characterize the endogenous ion channels and receptors present in this cell. The study of the role of ion channels is crucial to understand the processes of egg maturation, activation, fertilization, and meiotic progression. These events have already been explored in *X. tropicalis* [5,6]; however, there is a gap in the knowledge of the electrophysiological, molecular, and pharmacological properties of the oocyte ion conductances. Our laboratory has employed *X. laevis* oocytes for many years as a model system for heterologous expression of cloned receptors as well as for transplanting receptors and channels embedded in their own plasma membrane [7–11]. In addition, we have studied several

Abbreviations: 9-AC, anthracene-9-carboxylic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; ClC, voltage dependent chloride channels; DIDS, 4,4′-diisothiocyanatostilbene, 2,2′-disulfonic acid disodium salt hydrate; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; MS-222, 3-aminobenzoic acid methylester; NFA, niflumic acid; NPPB, 5-nitro-2(3-phenylpropylamino)benzoic acid

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membrane receptors and ion currents present in the oocyte, including Ca^{2+}- and voltage-dependent Cl^- currents [12–15].

Voltage-dependent Cl^- channels include the ClC anion channels/transporters, which constitute a gene family with at least nine members (ClC 1-7 and ClC Ka and Kb) [16]. In mammals, they play important roles such as cell-volume regulation, control of muscle excitability, and transepithelial transport. Several human diseases are known to be due to mutated ClC genes; for example, mutations in the muscle channel ClC-1 cause recessive (Becker) as well as dominant (Thomsen) myotonia [17], whereas X-linked hereditary hypercalciuric nephrolithiasis (Dent's disease) is due to mutations in the gene that codes for ClC-5 [18–20].

In the present study, we describe the characteristics of a ClC-like outward rectifying anion current in *X. tropicalis* oocytes (xtClC-or). This Cl^- current was clearly evident upon stepping the membrane potential to +45 mV; it presented higher permeability for anions such as SCN^- and I^- and was sensitive to extracellular pH. xtClC-or is mostly Ca^{2+}-independent, and the injection of an antisense oligonucleotide for ClC-5 blocked 50% of the xtClC-or current; the remaining current was also anion driven but, in contrast, was Ca^{2+}-dependent. This last component was found to be different from known Ca^{2+}-dependent Cl^- channels such as TMEM16A and bestrophins.

2. Material and methods

2.1. Oocytes and electrophysiological recordings

All the animals were handled in accordance with the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee of the National University of Mexico. *X. tropicalis* frogs were anesthetized with 0.1% ethyl 3-aminobenzoate methane sulfonate salt (MS-222) for 10 min. Follicles were manually removed, enzymatically defolliculated (with 0.3 μg/μl collagenase type I at room temperature for 35 min), and then kept at 16 °C in Barth's medium: 88 mM NaCl, 1 mM KCl, 0.33 mM $Ca_2(NO)_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 5 mM HEPES, pH 7.4, containing 0.1 mg/ml gentamicin sulfate. The electrophysiological recordings were obtained 24 h after isolation

Considering that *X. tropicalis* oocytes have an intracellular volume of 0.18 μl (calculated assuming the cells to be a sphere 0.8 mm in diameter), oocytes were injected with 5 nl of BAPTA or EGTA (600 mM) dissolved in RNAse-free water, which led to a final concentration of about 10.0 mM. This was done to prevent activation of Ca^{2+}-dependent currents. To ensure that 10 mM EGTA and/or BAPTA were buffering the cytosolic Ca^{2+} in *X. tropicalis* oocytes, we also tested the effect of a range of concentrations, from 5 to 100 mM, on the Ca^{2+}-dependent Cl^- current (T_{out}) [21]. To activate this current, the oocyte plasma membrane was held at −100 mV and stepped to +25 mV for 2 s and repolarized to −60 mV (*c.f.* Fig. 2A). Pipettes for EGTA and BAPTA injection were sterilized at 180 °C for 2 h and were mounted in a Drumont Nanoinjector (Drumont Scientific). After injection, oocytes were incubated at 16 °C in Barth's medium. The electrophysiological recordings were made 6–8 h after injection.

Oocytes were placed in a 300-μl chamber, impaled with two glass microelectrodes filled with 3 M KCl (0.5–1.5 MΩ). The oocyte's resting membrane potential was −30 ± 2 mV (N = 16, n = 90). A holding potential of −55 mV was used in all experiments. For the electrophysiological characterization of the outward current, current–voltage relationships were constructed by stepping the oocyte membrane potential from −105 to +95 in 20-mV steps; repolarization was to −85 mV or −105 mV for the experiments depicted in Fig. 6. All recordings were done at room temperature (20–23 °C) in a chamber continually perfused (5–10 ml/min) with frog's Ringer solution: 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.4.

In order to determine the extracellular pH (pH_e) sensitivity in the range of 4.5 to 9.5, the Ringer's solution was buffered with 5 mM MES for pH 4.5 or 6, and with 5 mM HEPES for pH 7.2, 8, and 9.5. All these solutions were titrated with 5 N HCl or 5 N NaOH. In ion substitution experiments, 58 mM Na^+ was replaced by $NMDG^+$, whereas 115 mM Cl^- was substituted by an equimolar concentration of aspartate, SCN^-, I^-, or Br^-. NPPB, DIDS, 9-AC, and NFA were dissolved in DMSO and added to the external solution to the final concentration indicated.

To determine the effect of suppressing the expression of ClC-3, ClC-5, ClC-7, and TMEM16A we injected 25 nl of sense or antisense oligonucleotides (2.5 μM) and assessed the effect 6 to 12 h later by running the activation protocol described above.

2.2. Drugs and statistics

All drugs were purchased from SIGMA. In all cases, the concentration of DMSO was 0.2%, which had no evident effect on the oocyte's membrane resting potential. Membrane currents are expressed as normalized current at +95 mV. Results are values obtained from at least 5 oocytes. Concentration–response curves were constructed by normalizing the currents to the maximum response evoked by the control at +95 mV. The half-inhibitory concentration (IC_{50}) of 9-AC, DIDS, or NFA was estimated by fitting the data to the following equation:

$$I = [(A_1 - A_2)/(1 + ([B]/IC_{50})^p)] + A_2.$$

In which I is the current amplitude, A_1 is the initial current value when no antagonist is present, A2 is the final current value at a saturating concentration of antagonist, [B] is the concentration of antagonist currently tested, p is the Hill exponent, and IC_{50} is the concentration of antagonist at which half of the maximum inhibitory effect is obtained.

In the case of the anion substitution experiments, it was assumed that the classical equation: $I = g(V - E_r)$ (where I is the current, V the membrane potential, E_r the reversal potential of the current, and g the macroscopic conductance) accurately describes the behavior of the outward rectifying current, as well as the lack of large variations in the term $(V - E_r)$ upon Cl^- replacement by other anions. If this assumption is true, then $I_X/I_{Cl} \cong g_X/g_{Cl}$ (where X represents other anions different from Cl^-, and I_X and g_X are the corresponding current and conductance values, respectively), and an apparent conductance sequence (relative to Cl^-) can be obtained from current amplitude ratios at a given membrane potential.

Differences among groups were evaluated by ANOVA ($P < 0.05$). Whenever an asterisk is present in a figure, it indicates a value significantly different from the control. Data are represented as the mean ± standard error of the mean (SEM). Control responses were elicited before and after each test condition to account for possible time- and/or activity-dependent changes.

2.3. RNA extraction, RT-PCR, and oocyte injection

Total RNA from defolliculated oocytes was obtained by a standard Trizol method (Invitrogen), followed by DNAse I treatment and heat inactivation. RNA integrity was confirmed by gel electrophoresis, and its concentration was measured by spectrophotometry. RT-PCR was performed using the SS®III One-Step RT-PCR kit (Invitrogen) using the following conditions: one step of 55 °C for 30 min, 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 40 s, and a final step at 68 °C for 2 min. PCR products were analyzed in a 2% agarose gel stained with ethidium bromide. Primers for ClC 2–7 and TMEM16A genes (Supplementary Table 1) were designed to

amplify specific fragments of each gene, according to the database JGI Eukaryotic genomics and on the corresponding predicted mRNA for *X. tropicalis* [4]; for ClC-1, primers were designed to align to highly conserved regions of this gene across species.

3. Results

3.1. Properties of the xtClC-outward current

The xtClC-or currents were recorded using the two-electrode voltage-clamp technique [12]. Fig. 1A illustrates a representative experiment showing the current response to voltage steps from −105 to +95 mV (in 20-mV increments). The holding potential was −55 mV, and when the membrane was depolarized from −105 to +55, an outward current was elicited. The current did not decay while the membrane was held at that voltage, and further voltage increases gave progressively larger currents. At potentials more positive than +15 mV, the current was outward in direction, and it increased almost linearly from +55 mV. This current practically did not inactivate, and a second depolarizing protocol, applied right after the end of the previous one, elicited currents of almost the same amplitude. The outward currents showed amplitudes in the range of 250 to 300 nA at +95 mV. To determine if this conductance was driven by Cl^- and to exclude the participation of Na^+, we followed two strategies: first we replaced Cl^- with aspartate (Cl^--free) and second, we partially substituted the Na^+ (50%) with $NMDG^+$. Full substitution of NaCl by $NMDG^+$ produced high membrane leakage, and the cells did not resist the voltage-clamp. Substitution of K^+ in the bath Ringer's was obviated because the reversal potential obtained was clearly far from the equilibrium potential for this ion. As shown in Fig. 1A–B, a drop of more than 70 ± 2.1% (ANOVA test, *P < 0.05) in the outward current was recorded at depolarizing potentials in Cl^--free Ringer's (n = 10, N = 3), while substitution of Na^+ by $NMDG^+$ did not affect the current. The current/voltage relationship shows that the current rectifies outwardly from +20 mV to more positive values. The reversal potential was around −13 ± 0.8 mV for the control, −10 ± 1 mV for $NMDG^+$, and +18 ± 0.9 mV for Cl^--free (Fig. 1B) (n = 10, N = 3, each). Similar outward currents are characteristic of some ClC anion transporters/channels such as ClC-4 and *X. laevis* ClC-5 [22,23].

3.2. Effects of intracellular and extracellular Ca^{2+}

To assess the potential role of intracellular $[Ca^{2+}]_i$ in the generation of the outwardly rectifying current, the oocytes were injected with 5, 10, and 100 mM (final concentration) of BAPTA or EGTA. In preparation for these experiments, we first determined whether these molecules were actually chelating intracellular Ca^{2+} by evaluating their effect on the well-known Ca^{2+}-dependent Cl^- current (T_{out}) found in frog oocytes [21]. Fig. 2A shows that 10 mM BAPTA or EGTA inhibited this current by 90 ± 1.4%, whereas 5 mM inhibited only 45 ± 2.5% of the current. When BAPTA or EGTA were used at 100 mM, most of the oocytes died within 1 h after the injection, and those that survived had unstable plasma membranes that prevented holding the potential to run the activation protocols; an example of oocytes that survived injection of 100 mM BAPTA or EGTA is shown in Fig. 2A. In conclusion, 10 mM BAPTA or EGTA was found to be optimal to assess the role of intracellular Ca^{2+} on the elicited currents.

To assess the effect of the chelating molecules on the xtrClC-or current, the oocytes were recorded in normal Ringer's solution (1.8 mM $[Ca^{2+}]_e$), and the current amplitudes recorded at +105 mV showed a diminution of 15 ± 2% and 20 ± 2.2% with BAPTA and EGTA, respectively (ANOVA test, *P < 0.05) (Fig. 2B–C) (n = 7, N = 5), most probably because of a contribution from an endogenous current that was resistant to 10 mM EGTA or BAPTA.

We also performed experiments in which normal extracellular Ca^{2+} (1.8 mM) was replaced by 10 mM Mg^{2+}. This substitution aimed to prevent Ca^{2+} influx from the extracellular medium because: 1: we have a nominally Ca^{2+}-free solution and, 2: voltage-operated Ca^{2+} channels are expected to be blocked by this concentration of Mg^{2+}. As shown in Fig. 2B–D, this treatment induces an even larger drop (in comparison with BAPTA or EGTA) in the amplitude of the outward rectifying current. Thus, in this case the outwardly rectifying current diminished by 26 ± 3.1% (ANOVA test, *P < 0.05) (Fig. 2B–D) (n = 9, N = 4). We argue that this effect is mainly unrelated to Ca^{2+} influx, but rather depends on other nonspecific effects of Mg^{2+}. We also observed that mere omission of $Ca^{2+}{}_o$ (in the absence of Mg^{2+}) has only a small, if any, effect on the outward rectifying current (data not shown).

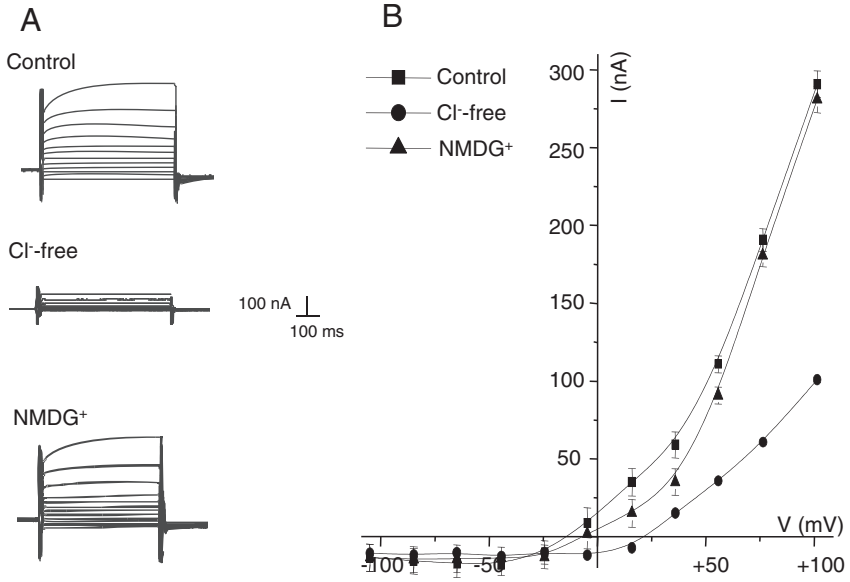

Fig. 1. Outward currents in *X. tropicalis* oocytes. A) Sample currents recorded from an oocyte in normal Ringer (control), equimolar concentration of aspartate (Cl^--free) or Na^+ 50% replaced by $NMDG^+$. Note the marked diminution of the outward current when Cl^- was replaced by aspartate, whereas when Na^+ was partially replaced by $NMDG^+$ no significant effect was detected. B) Current–voltage relations. Observe the reversal potential at −15 mV and −10 mV for the control and $NMDG^+$ media and +18 mV in Cl^- free media, indicating that Cl^- is the main ion passing through the channel (n = 10, N = 3). The holding potential was −55 mV; 500 ms test pulses were applied from −105 to +105 in 25-mV increments. Data points are means ± S.E.M.

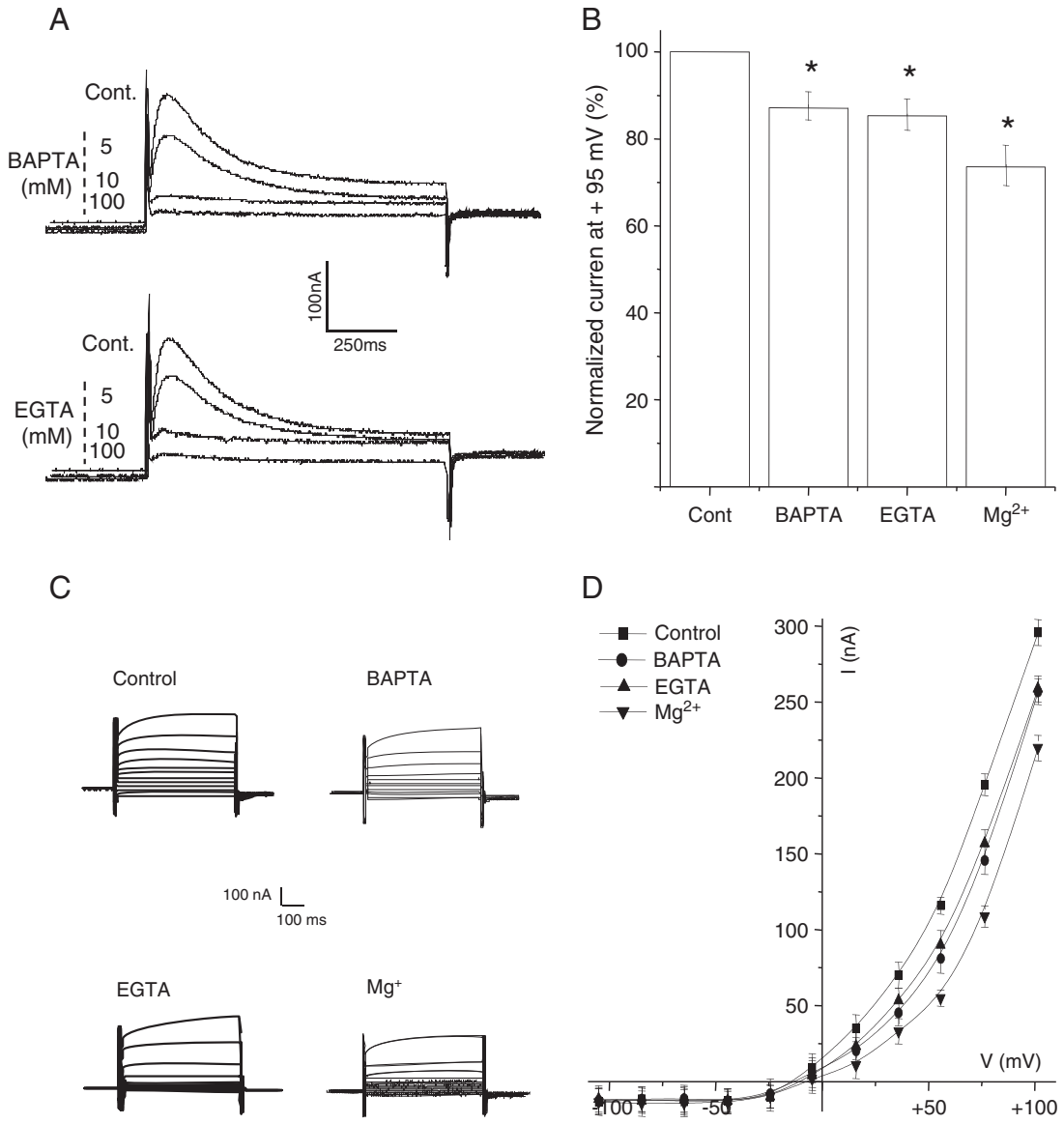

Fig. 2. Intracellular and extracellular Ca^{2+}-dependence of the outward current. A) Effect of injecting 5, 10, and 100 mM BAPTA or EGTA on the endogenous T_{out} current. Observe that at 10 mM, both compounds abolished the T_{out} current (90 ± 1.4% reduction) (n = 7, N = 2). B–C) Effect of the 10 mM BAPTA or EGTA on the outward current; note a 15–20% diminution of the current with either compound (n = 9, N = 4). The outward current with 10 mM Mg^{2+} was 26 ± 3.1% lower than in Ca^{2+}-free solution (n = 5, N = 3) (ANOVA test, * $p < 0.05$). D) Current–voltage relations. Note that none of these conditions shifted the reversal potential or showed an effect at different potentials. In B, the currents are expressed as percent of the control, and points are the means of several experiments. Data points are means ± S.E.M.

3.3. Pharmacology

To assess the pharmacological properties of the endogenous xtClC-or, we tested its sensitivity to several Cl^--channel blockers, including 9-AC, DIDS, NPPB, and NFA. Fig. 3A shows that only NPPB and NFA (each at 500 μM) inhibit reversibly the outward current by 75 ± 1.0% (n = 7; N = 2) and 65 ± 1.1% (n = 5; N = 2), respectively. In contrast, neither DIDS nor 9-AC exerted any effect at the concentrations tested that ranged from 50 to 500 μM. The IC_{50} values for NPPB and NFA were 300 ± 10 μM and 400 ± 12 μM, respectively (Fig. 3B).

3.4. Ion conductance and effect of extracellular pH

Since we could not determine a shift of the reversal potential for the anion substitution due to the absence of inward currents, derived from the transient opening of the channel, we did not determine an ion sequence based on permeability ratios for the outward rectifying current. Instead, the whole-cell conductance sequence was estimated from current amplitude ratios, as described for other outward ClC transporters/channels such as hClC-4 and *X. laevis* ClC-5 [22,23]. Fig. 4A–B shows the current amplitude at +95 mV in the presence of each anion. The resulting sequence was: $SCN^- > I^- > Br^- > Cl^-$, with current amplitude ratios of 1.9 ± 0.30:1.7 ± 0.39:1.4 ± 0.28: 1.0, respectively (n = 8, N = 3).

Several studies have reported that changes in pH modulate ClCs, and that H^+ is actually co-transported with Cl^- by some of these protein complexes [24]. Thus, we tested the effects of the extracellular pH (pH_e) on the xtClC-or current. Fig. 4C–D, shows that an acidic pH_e of 4.5 or 6.0 diminished the outward current by 57 ± 2.1% and 40 ± 1.1% respectively (ANOVA test, *$P < 0.05$) (n = 7, N = 3). In contrast, an alkaline pH_e induced a slight diminution in the outward

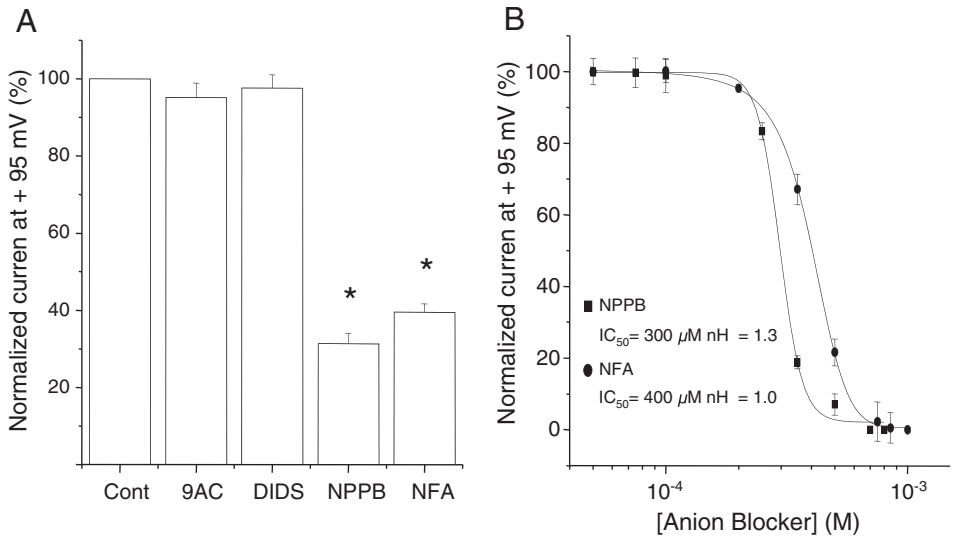

Fig. 3. Sensitivity of the outward current to chloride channel blockers. A) Effects of 9-AC, DIDS, NPPB, and NFA on the outward current; note that only NPPB and NFA blocked the current, by 75 ± 1.0% and 65 ± 1.1%, respectively. The data are reported as percent of the control (ANOVA test, * $p < 0.05$) (for both: $n = 5$; $N = 2$). B) The effects of NPPB and NFA were concentration dependent with an IC_{50} of 300 ± 10 μM for NPPB and 400 ± 18 μM for NFA. The currents are reported as percent of the control (for both: $n = 6$, $N = 3$). Data points are means ± S.E.M.

current of 6.5 ± 2% and 8 ± 1.2% for pH 8.0 and 9.5, respectively (ANOVA test, $P > 0.05$) ($n = 9$, $N = 3$). These effects were fully reversible upon return to normal Ringer's solution (pH 7.2). Under these experimental conditions we did not observe any evident change in the kinetics of the current or in the reversal membrane potential.

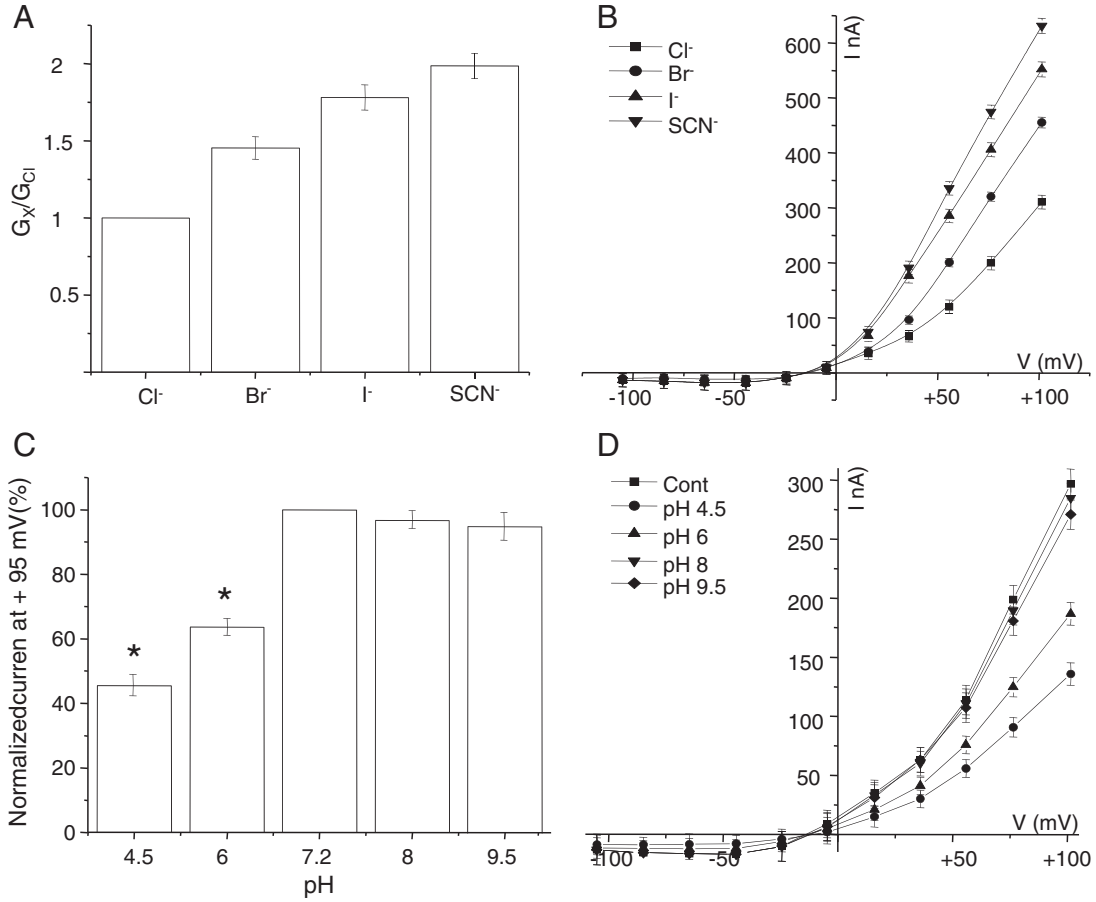

Fig. 4. Whole-cell conductance sequence and pH sensitivity. A) Current amplitude ratios obtained at +105 mV for each anion (I_X/I_{Cl} where x indicates various anions). Currents normalized to the current generated in the presence of Cl^- ($n = 8$, $N = 3$). B) Current–voltage relationships. Note the increased currents at the various potentials tested with Br^-, I^-, and SCN^-. C–D) pH dependence of the outward current. Note the negative modulation of the current at acidic values, by 40 ± 1.25% and 56 ± 1.0% with pH 4.5 and 6, respectively. In D the current amplitudes obtained at various extracellular pH values are reported as percent of maximum current amplitude measured at pH 7.2. Ringer media were buffered with MES or HEPES. ($n = 7$–9, $N = 3$) (ANOVA test, *$P < 0.05$). Data points are means ± S.E.M.

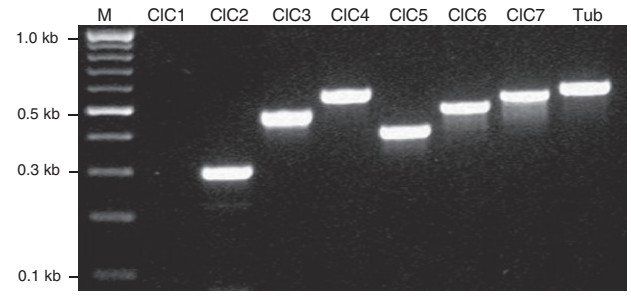

Fig. 5. mRNA expression analyzed by RT-PCR in *X. tropicalis* oocytes. Agarose gel stained with ethidium bromide demonstrating the presence of the mRNAs coding for ClC-2 to ClC-7 anion transporters/channels. The housekeeping gene tubulin (tub) was included as an internal control. A negative control without RNA was included for each primer set and produced no amplification bands (not shown).

3.5. Expression of the ClC mRNAs in X. tropicalis oocytes

To determine whether ClC mRNAs are expressed in *X. tropicalis* oocytes, RT-PCR assays were performed using as template RNA from isolated oocytes. The primers designed for this purpose are shown in Supplementary Table 1, and the predicted amino acid sequences for each *X. tropicalis* ClC are shown in Supplementary Fig. 1. The PCR detected the mRNAs of ClC-2 to ClC-7, whereas ClC-1 was not found (Fig. 5). It is known that several of these transporter/channel proteins, such as ClC-3 and ClC-5, are located in intracellular compartments as well as in the plasma membrane [25,26]. However, based on the biophysical, pharmacological, and selectivity characteristics of the outward current, it seems that the xtClC-or current is due to the expression of a ClC-5-like gene. Furthermore, some evidence indicates that ClC-5 reaches the plasma membrane and generates voltage-dependent currents [27,28].

3.6. Blocking the xtClC-or current by antisense oligos

Considering the electrophysiological characteristics shared between xtClC-or and ClC-5 [23,29], we assessed the effect of injecting antisense oligos for ClC-5 into *X. tropicalis* oocytes on the elicited outward current. Four groups of oocytes were injected as follows: group 1 was injected with a sense ClC-5 oligo, group 2 with an antisense ClC-5 oligo, group 3 with both oligos, and group 4 with water; 6 to 8 h after injection, the xtClC-or current was recorded. Unexpectedly, in most cases after 12–14 h the oocytes injected with antisense ClC-5 oligos died, whereas oocytes injected with sense oligos survived, at least for 48 h. Six to 8 h post-injection, the ClC-5 antisense oligo reduced the amplitude of the outward current by 56 ± 2.3% (Fig. 6A) (n = 8, N = 3), whereas the ClC-5 sense oligo induced no apparent modifications in the elicited currents, which were similar to those of the non-injected oocytes or those injected with water (data not shown). In addition, the xtClC-or conductance does not shift the reversal potential when Cl^- is replaced by SCN^- and does not generate tail currents upon repolarization of the plasma membrane (see below). These results indicate that the ClC-5 transporter/channel is the most likely candidate generator of the xtClC-or current. Further experiments were done by injecting antisense oligonucleotides complementary to ClC-3 and ClC-7, but we did not observe any inhibition of the outward current (not shown).

The oocytes injected with the ClC-5 antisense oligo had a "remnant current" with a reversal potential of -13 ± 0.9 mV, while that of the control was −14 ± 1.2 mV (n = 8, N = 3) (Fig. 6B). In most of the recorded oocytes, these currents did not show a tail after repolarization of the plasma membrane, indicating that these do not correspond to conventional TMEM16A, and this was the case whether the oocyte was bathed in normal Ringer's buffer or in buffer with Cl^- replaced by SCN^- (Fig. 6C–D). Nevertheless, we used RT-PCR to test whether this channel is expressed in oocytes. Fig. 7A shows the amplified product that was detected in all the oocyte samples tested, revealing that TMEME16A mRNA is indeed present in oocytes. This is consistent with the T_{out} currents observed in *X. tropicalis* oocytes (Fig. 2A) and with the current typically generated by this channel in *X. laevis* oocytes [21]. However, injection of antisense TMEM16A in oocytes did not block the outwardly rectifying current (Fig. 7B–C), which was partially blocked by an antisense ClC5 (Figs. 6 and 7B–C).

The remnant current after anti ClC-5-injection showed a 70 ± 1.3% decrease when $[Ca^{2+}]_e$ was substituted by Mg^{2+} (10 mM) (n = 10, N = 4), and a decrease of 90 ± 2.1% with BAPTA and EGTA (10 mM) (n = 10, N = 5) (ANOVA test, *P < 0.05) (Fig. 8A–C). This "remnant current" differs from xtClC-or in antagonist sensitivities; i.e., it was DIDS insensitive and sensitive to NPPB, NFA and 9-AC with an inhibition of 80 ± 3.2%, 57 ± 2.1%, and 45 ± 3.0%, respectively (ANOVA test, *P < 0.05) (n = 6, N = 4) (Fig. 9A–B). The whole-cell conductance sequence of this current was: $SCN^- = I^- > Br^- > Cl^-$ with current amplitude ratios of 2.3:2.5:1.7: 1, respectively (n = 9, N = 3) (Fig. 9C–D).

4. Discussion

Several endogenous Cl^- currents have been characterized in *Xenopus* oocytes including Ca^+-activated ($I_{Cl,Ca}$) and voltage-activated ($I_{Cl,H}$) currents [12,30–33]. Thus far, several Cl^- channels have been cloned from amphibians: the *Xenopus* homologue of CFTR [34], ClC-K [35], ClC-3 and ClC-5 [36], bestrophins [1], and TMEM16A [37].

In this work we determined the properties of an outwardly rectifying anion current of *X. tropicalis* oocytes that we have denominated xtClC-or. This current shares some characteristics with ClC transporters/channels such as the ClC-5 of several species [23,27,28,38–41]. xtClC-or was partially independent of $[Ca^{2+}]$, and its sensitivity to several Cl^- channel blockers was peculiar. For example, it contrasts with the *X. laevis* ClC-5 which is insensitive to DIDS, NPPB, 9-AC, and NFA [27,38], whereas xtClC-or was insensitive to DIDS and 9-AC but was reversibly blocked by NPPB and NFA. Interestingly, some properties of the xtClC-or, such as voltage-dependence, strong outward rectification, and no inward currents are similar, but not identical, to the properties of the voltage-dependent Cl^- transporter/channel ClC-5. However, there are clear differences in anion selectivity and pharmacological profile. The anion selectivities of xClC-5 and hClC-5 are $NO_3 > Br^- > Cl^- > I^- \gg$ gluconate and $NO_3 > Cl^-$ > bicarbonate > $I^- \gg$ glutamate, respectively [38,42], whereas that of xtClC-or is $SCN^- > I > Br^- > CI^-$.

One characteristic conserved among ClC-5 orthologs is their modulation by extracellular pH. In our study, xtClC-or was reversibly inhibited by acidic pH, in accordance with xClC-5 and other species [27,28,39,42]. Furthermore, xtClC-or showed only little sensitivity to basic extracellular pH a property consistent with ClC-5, which is insensitive to pH from 7.5 to 9.5 [28].

RT-PCR revealed the expression of ClC-2 to ClC-7 mRNAs in *X. tropicalis* oocytes. However, the electrophysiological characteristics of xtClC-or and the fact that the ClC-5 antisense oligo reduced the current, (although it did not completely abolish it) strongly suggest that ClC-5 is the major component of xtClC-or in *X. tropicalis* oocytes. Different studies have shown that ClC-5 of *X. laevis* is largely located in intracellular compartments and does not significantly contribute to the membrane conductance in these cells [39,41]. However, our results showed a strong, outwardly rectifying anion current indicating the possibility that xtClC-5 could be present in the plasma membrane of the *X. tropicalis* oocyte. Evidence suggests the presence of ClC-5 in the plasma membrane of the *X. laevis* renal epithelial A6 cells [27]. Moreover, when ClC-5 is heterologously expressed in COS-7, HEK,

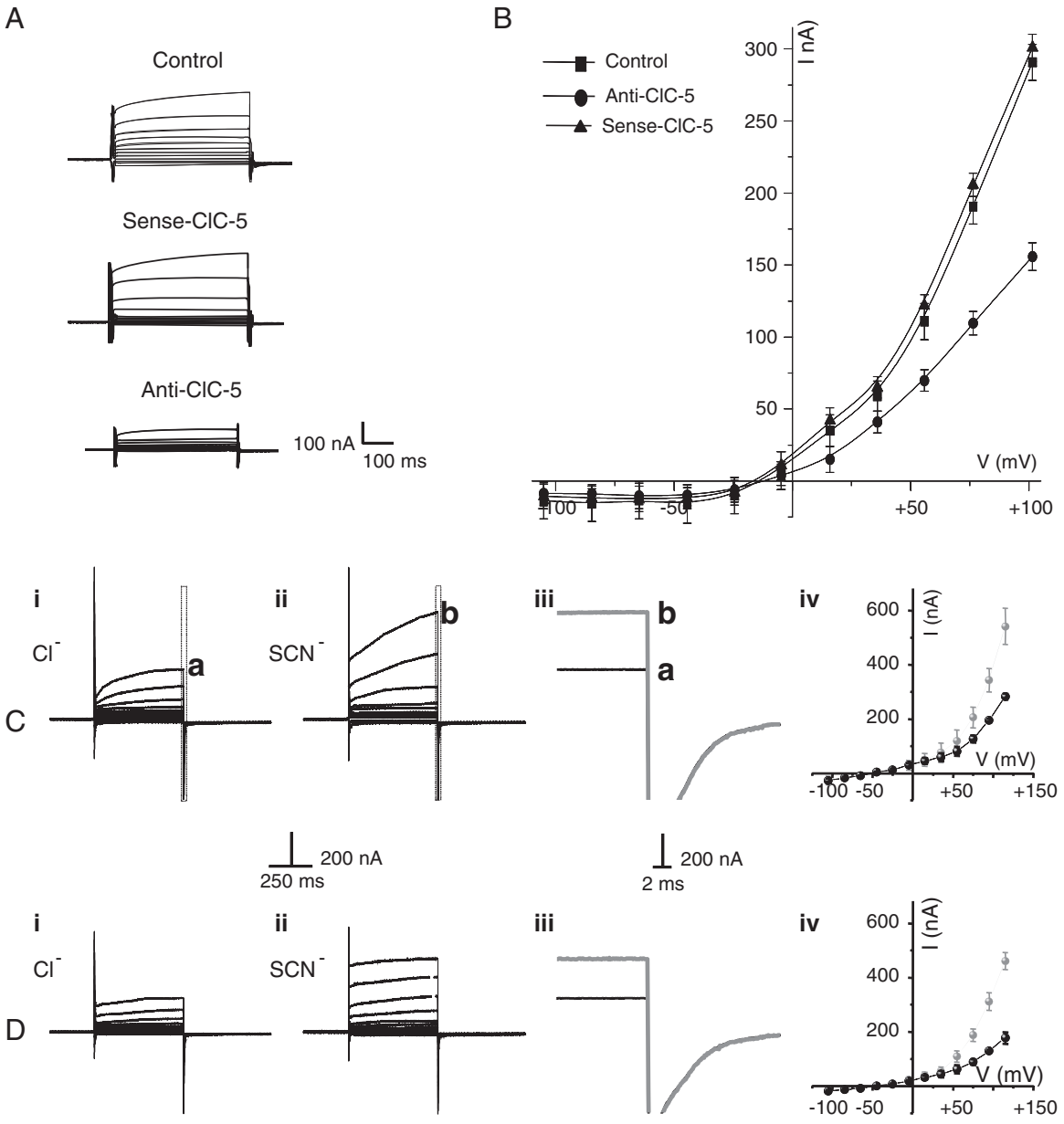

Fig. 6. Injection of ClC-5 antisense oligonucleotide. Observe that ClC-5 antisense partially abates the outward current, and the remaining current displays neither a shift in reversal potential upon anion substitution, nor tail currents. A) Representative currents recorded from oocytes 8 h after injecting the sense or antisense oligo for ClC-5. Note the reduction by 56 ± 2.3% in the current amplitude in oocytes 8 h after injection of the antisense sequence. B) The voltage–current relationships showed a "remnant current" in the presence of antisense ClC-5. This current had a reversal potential of −13 ± 0.9 mV (n = 8, N = 3), while that of the control and the ClC-5 sense oligo were −14 ± 1.2 mV and −13 ± 1 mV, respectively (n = 6–8, N = 3–4). The holding potential was −55 mV; 500-ms test pulses were applied from −105 to +115 in 20-mV increments. In C) and D) oocytes were injected with either water (C) or ClC-5 antisense oligonucleotide (D). From a holding potential of −55 mV, 500-ms voltage steps were applied from −105 mV to +115 mV in 20-mV increments, followed by a 500-ms step to −115 mV. Oocytes were bathed in the normal (115 mM Cl^-) solution (i) and in a solution with Cl^- replaced by an equimolar concentration of SCN^- (ii). (iii) Boxed regions are shown in more detail for cells bathed in Cl^- (a, black trace) and SCN^- (b, gray trace) in the transition from the +105-mV step to the −105-mV step. Note that no substantial tail current is observed in any case, regardless of the main extracellular anion present. (iv) Current–voltage curves obtained with oocytes bathed in Cl^- (black) and SCN^- (gray) external solutions. Note that no shift in reversal potential is observed upon substitution of Cl^- with SCN^- (n = 6, N = 2, for both C and D). Data points are means ± S.E.M.

and CHO-K1 cells, a fraction of the expressed transporter/channel population is targeted to internal organelles while another fraction reaches the plasma membrane, where it forms voltage-activated Cl^- channels [23,27,38,42]. The possibility of the formation of heterodimeric ClCs in the frog oocytes remains to be explored, and this may explain the peculiar pharmacology of xtClC-or; however, injection of antisense oligonucleotides to ClC3 and ClC-7 did not block the current.

In *X. laevis* oocytes, in which a very similar, outwardly rectifying conductance has been described, chelation of $Ca^{2+}{}_i$ with BAPTA decreased the outward current amplitude to nearly the same extent as described here, and a lack of tail current is a distinctive feature of this conductance [29]. Thus, oocytes from these closely related frog species behave pretty much the same with regard to this current. In addition, we provide evidence that TMEM16A is expressed in *X. tropicalis* oocytes, which may explain the endogenous T_{out} (Fig. 2) and the oscillatory Ca^{2+}-dependent Cl^- current described by Marchant and Parker in these oocytes [43].

Whether the similarity of the outward rectifying current between the two species of *Xenopus* is due to the expression of the same molecular repertoire, remains to be investigated. A previous report

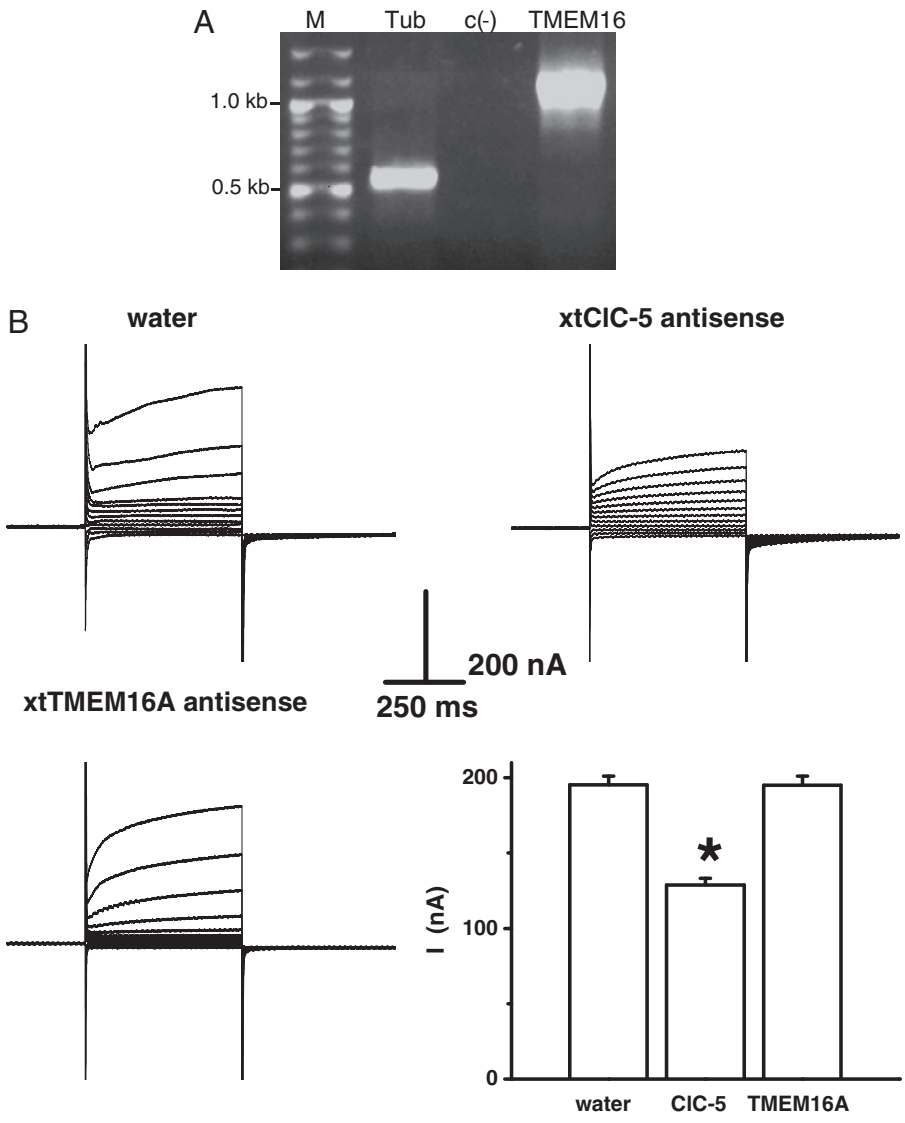

Fig. 7. TMEM16A is expressed in *X. tropicalis* oocytes but does not contribute significantly to the outward current. A) TMEM16A mRNA expression identified by RT-PCR in *X. tropicalis* oocytes. Tub, Tubulin; c(−), negative control. B) Oocytes were injected with water, xtClC-5 antisense oligo, or xtTMEM16A antisense oligo (30 ng per oocyte) and recorded after 12 h. From a holding potential of −55 mV, 500-ms voltage steps were applied from −105 mV to +115 mV in 20-mV increments, followed by a 500-ms step to −105 mV. Amplitude of the current (at the end of the +95-mV voltage step) is plotted for each group of injected oocytes. *Denotes significant difference ($P < 0.05$). n = 6, N = 2 for each group. Data are presented as means ± S.E.M.

suggests that even though ClC-5 is endogenously expressed in *X. laevis* oocytes, it is mainly targeted to intracellular organelles and makes no significant contribution to plasma membrane currents [27]. Thus, in comparison with *X. laevis* oocytes, xtClC-5 may be more actively and persistently targeted to the plasma membrane than ClC-5 in *X. laevis* oocytes.

It is possible that the Cl^- conductance deficit arising from the knock down of ClC-5 transporters/channels could be slightly compensated by over-expression of other Cl^- conduction pathways, particularly the residual Ca^{2+}-dependent outward rectifying conductance disclosed in this study. In this regard, it is interesting to observe that acute treatment with either BAPTA or EGTA only abolished ~30–50 nA of current (Fig. 2, Vm = +95 mV) in control oocytes (not treated with ClC-5 antisense oligo), while the Ca^{2+}-dependent residual current is ~150 nA (Fig. 6, Vm = +95 mV) in oocytes treated with the antisense. Expression of TMEM16A mRNA was detected in all the samples of oocytes tested; however, it is unlikely that the remnant conductance described in the present work could be generated by the 'classical' CaCCs for the following reasons: 1: No tail current was observed in the remnant current, while CaCCs display notorious, exponentially decaying tail currents [44]. 2: Anions such as SCN^- or I^- display higher conductance than Cl^- in the remnant outward rectifying current conduction pathway, while it is known that $G_X/G_{Cl} < 1$ in the case of CaCCs (with X denoting SCN^- or I^-, both of which are more permeable but less conductive than Cl^-) [45]. 3: DIDS does not block the remnant outward rectifying current conductance, while it is a highly effective blocker of CaCCs [46]. 4: Even though CaCCs display moderate outward rectification (especially at low $[Ca^{2+}]_i$), this rectification is not as strong as the one presented in this work. Thus, if CaCCs underlie the remnant outward rectifying current conductance, clear shifts in reversal potential would be observed upon application of the voltage-step protocol in conjunction with Cl^- replacement by a more permeable anion (e.g., SCN^-). But this is clearly not the case, as shown in Fig. 9D. 5: Injection

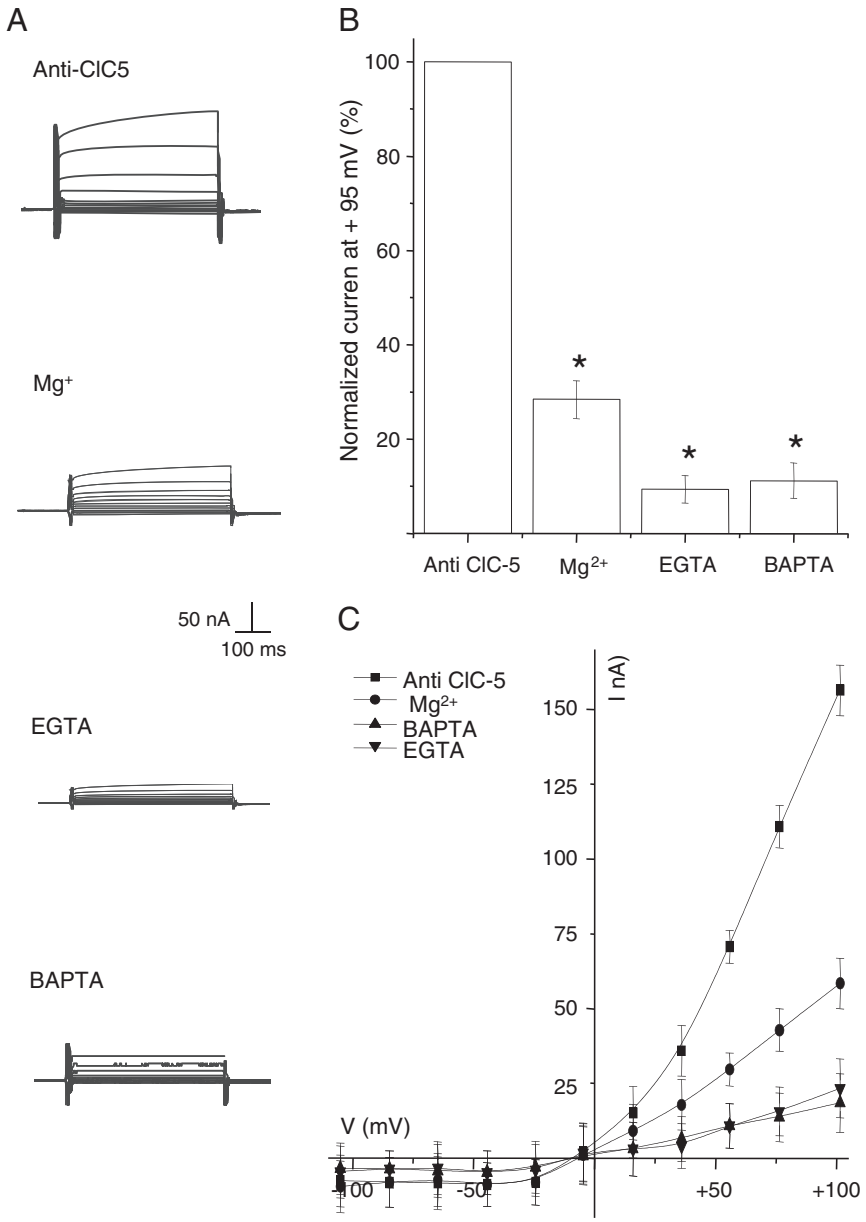

Fig. 8. Ca^{2+} dependence of the "remnant current". The experimental conditions were the same as for Fig. 2. The oocytes were injected with the ClC-5 antisense oligo 6 h before BAPTA or EGTA injection. A) Sample currents of oocytes injected with either anti-ClC5 oligo, 10 mM EGTA, or 10 mM BAPTA; effect of replacing Ca^{2+} by Mg^{2+} in Ringer's buffer. B) Note the 90 ± 2.1% drop in current amplitude with EGTA and BAPTA (ANOVA test, * $p < 0.05$) ($n = 10$, $N = 5$). Replacing Ca^{2+}-free by 10 mM Mg^{2+} reduced the outward current by 70 ± 1.0% ($n = 10$, $N = 4$). C) Current–voltage relations after injecting BAPTA or EGTA, or after substitution of Ca^{2+} by Mg^{2+}. The currents are reported as percent of the control. In B the control points are the means of absolute current. Data points are means ± S.E.M.

of antisense TMEM16A did not affect the outwardly rectifying current, thus suggesting that this channel does not contribute in a major way to the xtClC-or.

Thus, this remnant conductance is most probably mediated by ion channels other than CaCCs or, alternatively, it could depend on carrier molecules, as suggested by the lack of tail currents. Interestingly, it was recently reported that another member of the TMEM16/anoctamin family, TMEM16F/ANO-6, underlies the remnant outward rectifying current conductance in airway epithelial cells and Jurkat T cells [47] and has also been linked with Ca^{2+}-dependent scramblase activity in platelets [48]. Hence, it is possible that TMEM16F could act as a membrane carrier which couples Cl^- efflux with movement of lipids in a Ca^{2+}-dependent manner. Further research is needed to test this hypothesis.

In summary, we demonstrated the expression of six ClC mRNAs in *X. tropicalis* oocytes, and found evidence that the ClC-5 ortholog induces the strong outward rectification current characterized in this paper. In addition, we showed that this current has a different pharmacological profile from the known *X. laevis* ClC-5 [26,38]. More experiments are needed to elucidate the possible interaction of xtClC-5 with other endogenous channels and to explain the functional relevance of such voltage-dependent ion currents in oocytes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.03.013.

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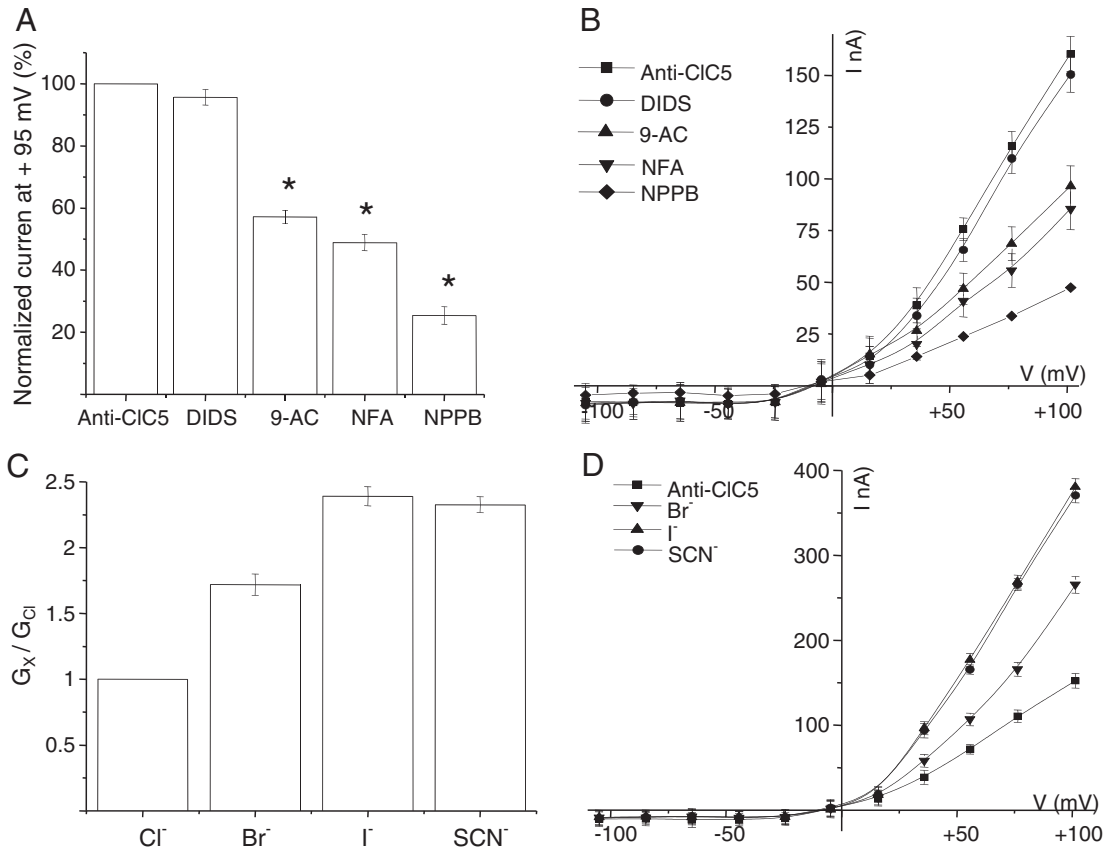

Fig. 9. Pharmacology and ion selectivity of the "remnant current". A, B) Effects of chloride channel blockers. Unlike xtClC-5, this current was inhibited by 9-AC, NFA, and NPPB by 45 ± 3.0%, 57 ± 1.1%, and 80 ± 3.2%, respectively (ANOVA test, *P < 0.05,). C–D) Whole-cell conductance sequence. Current amplitude ratios were obtained at +101 mV (G_X/G_{Cl} where x indicates the various anions) and normalized to the current obtained in the presence of Cl^- (n = 9, N = 3). Data points are means ± S.E.M.

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